Crucian carp heart performance during anoxia and acidosis

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Abstract

The crucian carp (*Carassius carassius* L.) exhibits the unique ability among vertebrates to maintain cardiac performance at normoxic levels during prolonged anoxia exposure. This thesis investigates the hypothesis that this phenomenon is possible because the heart likely never experiences an extracellular pH below 7.4 due to the fish's exclusive trait of converting lactate and H^+ ions to ethanol. Performance of isolated spontaneous beating heart preparations from crucian carp was measured during exposure to 100 min of anoxia at pH 7.8 and graded acidosis (between pH 7.8 and 7.0) under both normoxia and anoxia at 6.5^0C. As expected, the results revealed that anoxic performance of the crucian carp heart is severely affected by a fall in pH below the normal anoxic level of 7.4. This suggests that in nature, heart performance may not be able to meet the needs of the body if blood pH is not maintained above 7.4. Additionally, the ability for these isolated hearts to recover from 40 min anoxia exposure at different pH (7.8, 7.4 and 7.0) was investigated in both crucian carp and in anoxia-intolerant koi carp (*Cyprinus carpio* L.). The latter hearts appeared irreversibly damaged by anoxia exposure despite showing a similar anoxic depression of cardiac performance as crucian carp hearts. Thus, the crucian carp heart has apparently an innate ability to tolerate anoxia, which the koi-carp hearts lacks, an ability that most likely reflect that the crucian carp has evolved to tolerate long-term anoxia supported by ethanol production.

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Abbrevations

AA: the anoxia and acidosis group

AP: action potential

AC: the anoxic control group

ATP: adenosine trisphosphate

bpm: beats per minute

f_H: heart rate

F_{max}: maximum isotonic force

NA: the normoxia and acidosis group

NC: the normoxic control group

PC: pumping capacity

PO: cardiac power output

TPT: time to peak tension

TN-*C*: troponin-*C*

$T_{1/2R}$: time to half relaxation

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1 Introduction

1.1 The need for molecular oxygen

The evolution of the enormous variety of animal-life to be found on this planet could not have taken place without molecular oxygen produced by photosynthetic life forms. Oxygen is the terminal electron acceptor in the metabolic pathway of cellular oxidative phosphorylation that produces the universal energy molecule adenosine trisphosphate (ATP). The production of ATP is most efficient when carbohydrate, lipid or amino acid fuels are completely catabolized to CO_2 and H_2O in the presence of oxygen. When there is oxygen available (normoxia), the oxidation of 1 mol glucose can theoretically produce 36 mol ATP. In comparison, the only sustainable pathway for ATP production when oxygen is unavailable is glycolysis, where the end-product is normally lactate and only 2 mol ATP per mol glucose can be produced. In practice, glycolysis has an ATP yield that is approximately 10% of oxidative metabolism, as mitochondrial coupling during normoxia is rarely 100 % (Hochachka and Somero, 2002). As a physiological response to anoxia (no oxygen), most animals boost glycolytic capacity as they attempt to balance ATP economy. However, an up-regulation of glycolysis leads to detrimental lactate accumulation and metabolic acidosis. Further, despite the up-regulation, ATP demand still exceeds ATP production during anoxia for most animals, leading to death within minutes.

1.2 The aquatic anoxia challenge

Water breathers have not as easy access to O_2 as air breathers due to the low oxygen solubility in water compared to air. On the other hand, water breathers generally do not have a problem with respiratory acidosis because carbon dioxide easily dissolves in water. However, the resultant low blood carbonate levels mean that water breathers have about $1/10^{th}$ of the blood bicarbonate buffer capacity of air breathers and are not well adapted to acidosis (Rahn and Garey, 1973). Consequently, fish readily experience acidosis in hypoxia (Bennett, 1978). In some warm eutrophic ponds, decomposers' oxygen use exceeds the oxygen production of photosynthetic plants and algae, and the water becomes gradually anoxic. In the Nordic winter, some ponds are ice-capped for several months, which physically blocks the water from air contact, and even light, so little or no photosynthesis takes place (Fig. 1) (Vornanen

and Paajanen, 2004). Such aquatic habitat represents a selective force for animals to evolve mechanisms that allow survival during hypoxia and anoxia.

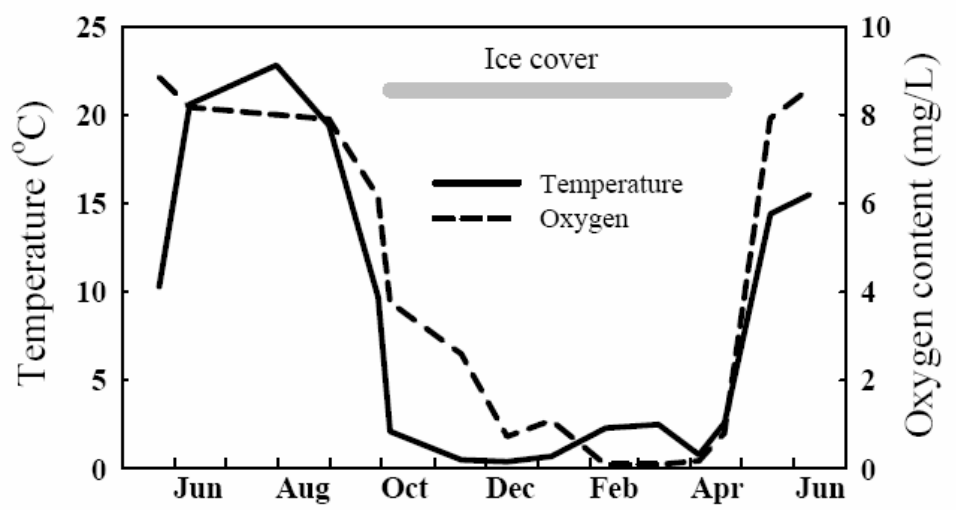

Fig. 1: Annual temperature and oxygen fluctuation of a pond in Finland (Vornanen and Paajanen, 2004).

1.3 Strategies for anoxic survival

Some animals can survive for several months in anoxic conditions under ice covered ponds (Blazka, 1958; Ultsch and Jackson, 1982). Interestingly, there are different metabolic ways to deal with the situation and the main two strategies are (1) to down-regulate metabolism (hypometabolism) or (2) to up-regulate glycolytic rate (Pasteur effect). In both strategies the purpose is to match the amount of ATP used to ATP produced. Some freshwater turtles (genera *Trachemys* and *Chrysemys*) drastically suppress metabolism and cardiac activity, and enter into a comatose-like brain state to decrease ATP consumption (Fernandes et al., 1997; Hicks and Farrell, 2000). It has been shown that the metabolic rate in turtles is depressed to less than 10% of normoxic rate at 12 weeks of anoxia exposure at 3^0C (Herbert and Jackson, 1985a). To cope with acidosis, lactate and hydrogen ions are buffered by the turtle's shell and bone (Davis and Jackson, 2007; Jackson, 2004).

Another anoxic survivor is the crucian carp (*Carassius carassius* L.). It has been found to survive anoxia for 5.5 months in natural ponds (Blazka, 1958) and for at least 4.5 months in controlled anoxic conditions (Piironen and Holopainen, 1986). What is really spectacular is that the crucian carp maintains brain activity (Nilsson, 2001; Nilsson et al., 1993), keeps up

cardiac performance (Stecyk et al., 2004), and even performs protein synthesis (Smith et al., 1996) and mitosis (Sollid et al., 2005a) during prolonged anoxia. Even if the locomotion is reduced by 50%, the physically active state has been suggested to be beneficial in the way that the carp does not have to wait to be reached by oxygen diffusion in the spring, but can shorten the anoxic period by moving to better oxygenated areas (Nilsson et al., 1993). This makes the crucian carp the real champion of anoxic survival. In many places, it is the only fish that can inhabit small seasonally-anoxic ponds. Globally, it is now to be found in central Asia and Europe, ranging from the north of Scandinavia to the south Black Sea and from England in the west to Russia in the east (Blazka, 1958; Holopainen et al., 1997b).

1.4 General anoxic survival strategies of the crucian carp

Of the two anoxic survival strategies detailed above, the crucian carp utilize some of both. To balance energy demands to maximum glycolytic capacity the carp depresses metabolic activity as evidenced by a 70% suppression of whole-body heat production (Van Waversveld et al., 1989). Also, protein synthesis is down-regulated by 50% in heart and skeletal muscles, and by 95% in the liver, where as it is unchanged in the brain (Smith et al., 1996). The anoxic crucian carp even shuts down its retina and suppresses hearing (Johansson et al., 1997; Suzue et al., 1987). Still the speed of glycolysis needs to be up regulated in brain by an estimated 2.4-times to balance ATP supply to demand (Johansson et al., 1995).

During late summer and autumn the crucian carp accumulates a huge store of glycogen (Holopainen et al., 1997a) where the main reservoir is to be found in the liver, which can contain up to 30% glycogen wet weight equaling 4.5% of whole body mass (Hyvarinen et al., 1985). In addition to liver glycogen, the white muscles can store glycogen up to 4%, the brain 3.3% and heart 8% of wet tissue weight (Hyvarinen et al., 1985; Rovainen, 1970; Vornanen, 1994; Vornanen and Paajanen, 2004; Vornanen and Paajanen, 2006) (Fig. 2). During the winter, the crucian carp does not eat (Penttinen and Holopainen, 1992) and the stored glycogen is the only available energy for use and probably sets the limit of anoxia survival time (Nilsson, 1990). In nature, about 20% of all this glycogen is still present by April which illustrates the crucian carps extreme capacity for long anoxic survival (Holopainen and Hyvarinen, 1985).

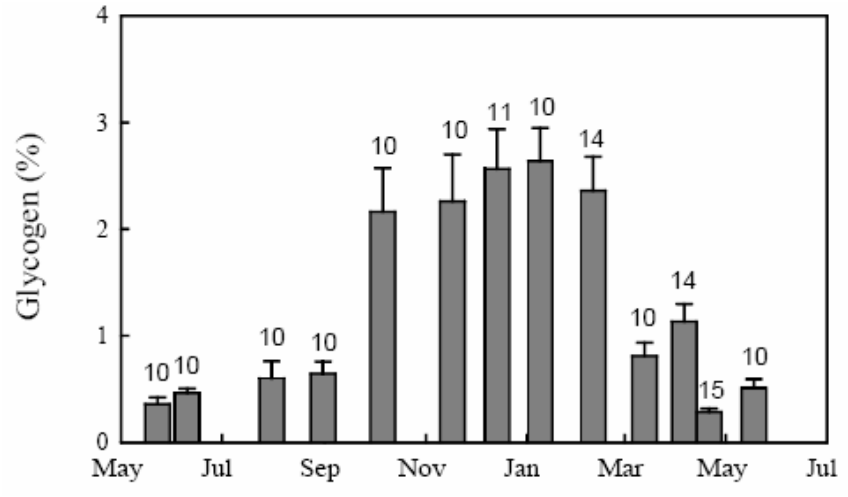

Fig. 2: Seasonality of cardiac glycogen stores in crucian carp. Similar fluctuations are seen in other tissues that store glycogen. (Vornanen and Paajanen, 2004)

Such high and prolonged glycolytic rate would kill most animals by metabolic acidosis (Nilsson and Lutz, 2004). A prerequisite for the ability of crucian carp to survive anoxia in an active state is its exotic ability to convert lactate to ethanol as the major end-product of anaerobic metabolism. The production of ethanol allows the fish to avoid poisoning by lactate and H^+ accumulation (Johnston and Bernard, 1983). This trait is only found in two other vertebrates closely related to the crucian carp, the goldfish (*Carassisus auratus* L.) (Shoubridge and Hochachka, 1980) and the bitterling (*Rhodeus amarus* L.) (Wissing and Zebe, 1988). The ethanol producing chemical pathway is performed in three enzymatic steps (Van Waarde, 1991) where (1) lactate is converted to pyruvate be the enzyme lactate dehydrogenase (LDH). Next, (2) pyruvate is converted to acetaldehyde by the Enzyme 1 unit (pyruvate decarboxylase) of the pyruvate dehydrogenase (PDH) complex. Finally, (3) the enzyme alcohol dehydrogenase (ADH) converts acetaldehyde to ethanol. ADH is only shown to exist in skeletal muscles, both red and white, but not in heart (Mourik et al., 1982; Nilsson, 1988). The lactate and ethanol trafficking to muscles is enhanced by a decrease in ventral aortic blood pressure and peripheral resistance of respectively 30% and 40%, respectively, which indicate a signifying vasodilation in peripheral tissue (Farrell and Stecyk, 2007; Stecyk et al., 2004). Ethanol toxification is avoided by diffusion of ethanol through the gills to the ambient water (Holopainen et al., 1986).

Another important strategy during hypoxia is to extract as much oxygen from the water as possible before it progressively becomes totally anoxic. A unique trait, observed in both

crucian carp and goldfish, is their ability to reversibly remodel an interlamellar cell mass (ILCM) between the gill lamella, which results in a 7.5-fold increase in respiratory surface area to maximize oxygen uptake (Sollid et al., 2003; Sollid and Nilsson, 2006). Further, the crucian carp red blood cells contain at least five hemoglobin isoforms with similar high oxygen affinities and similar Bohr shifts (P_{50} = 0.8 and 1.8 at pH 7.6 at10 and 20°C, respectively) (Sollid et al., 2005b).

1.5 The anoxic crucian carp cardiac survival strategy

The maintained cardiac activity in anoxic crucian carp is probably needed to transport glucose from the liver to the tissues, transport lactate to skeletal muscles for ethanol production, and for moving the ethanol to the gills for excretion (Stecyk et al., 2004).

The heart is a special challenge for anoxic survival due to its high intrinsic rate of ATP use that greatly exceeds that of most other tissues, except the brain (Stecyk et al., 2008). The crucian carp, like most fish, has a type 1a ventricle (Tota, 1989). The ventricle consists of only spongiosa tissue without capillaries and coronary arteries, which contrasts with more actively swimming teleost species that in addition often have an outer compact layer with coronary arteries (Farrell et al., 1992). Thus, the crucian carp heart only receives venous blood returning from the body which means it has to sustain its activity on low oxygen levels.

The heart requires ATP both for maintaining ion gradient, primarily kept up by Na^+/K^+-ATPase and Ca^{2+}-ATPases, and for running myofilament sliding, powered by myosin ATPases. Most hearts exposed to anoxia will quickly face an inadequate matching of glycolytic cardiac ATP supply with ATP demand, and meet death. Remarkably, the crucian carp's anoxia tolerant heart is able to maintain full cardiac activity for at least 5 days of anoxia in 8^0C (Stecyk et al., 2004).

Cardiac power output (PO; estimated from the product of cardiac output and central arterial pressure) is an indirect measure of cardiac ATP demand (Farrell and Stecyk, 2007). One factor that should facilitate the extraordinary cardiac performance of the crucian carp is its low ventral aortic blood pressure (1.7 kPa at 8^0C) which results in a low normoxic routine PO (0.46 mW g^{-1}). This PO is below what has been measured as the maximum anoxic capability of the anoxia-intolerant heart of rainbow trout (*Onchorhynchus mykiss* L.) (0.7 mW g^{-1}), which indicates that the crucian carp heart's ATP demand likely lies within the range of its maximal glycolytic potential, thereby allows it to maintain normoxic performance also in

anoxia (Farrell and Stecyk, 2007).

1.6 The problem with acidosis

Acidosis is detrimental for most hearts in a several different ways.

(1) First, extracellular acidosis directly affects pace maker cells in their ability to generate repeated AP's by slowing down self-depolarization via a reduction of inward ionic currents (Satoh and Hashimoto, 1983; Severi et al., 2002).

(2) Second, when intracellular hydrogen ion concentration increases, H^+ competes with Ca^{2+} in their binding to troponin (TN-*C*) of the contractile apparatus (Gesser and Poupa, 1983). The less charged H^+ do not cause the proper conformation change of TN-*C* resulting in less available actin seats for myosin motor proteins leading to decreased intrinsic contractile force.

(3) The third problem with acidosis is that the rise in intracellular H^+ concentration hinders the cardiac cells ability to produce the ATP needed for pumping Ca^{2+} over the sarcolemma (Marieb et al., 2006; Williamson et al., 1976). The intracellular increase in Ca^{2+} and H^+ also causes some gap junctions, which usually are open, to close, thereby electrically isolating the damaged cells and forcing the depolarization to take alternative routes. The pumping activity of the heart as a whole may be severely impaired.

(4) The forth negative effect of acidosis is that it depresses glycolysis by increasing the affinity for ATP of the inhibitory side of phosphofructokinase (Ui, 1966).

(5) The fifth consequence of extracellular acidosis occurs at the protein level. Some amino acids in a polypeptide chain have specific isoelectric points corresponding to the pH value were equal amounts of carboxy and amino groups are protonated or deprotonated (Nelson and Cox, 2005). This contributes to the pH sensitivity of an enzyme, which affect affinity to substrates. Thus, ion channel function can be severely impaired during acidosis.

(6) A sixth consequence of anoxia induced acidosis is apoptotic cell death (Graham et al., 2004; Hochachka et al., 1996). As studied in mammalian cardiomyocytes, an accumulation of hypoxia-inducible factor-1 (HIF-1) induces transcription of the apoptotic *BNIP*3 gene. The death inducing protein *BNIP3* will, in acidosis, translocate to mitochondria were it stimulates the opening of a permeability transition pore. The subsequent release of cytochrome-*c* and

calcium stimulates proteases and DNases to perform apoptotic cell death.

1.7 The objective of this thesis

The crucian carp is capable of prolonged anoxic survival in an active state relying on its metabolic suppression, glycolytic up-regulation, a low routine heart PO, a high blood O_2 affinity, and the exclusive ethanol pathway. Due to the ethanol production the anoxic crucian carp probably never experiences severe acidosis. Indeed, previous research has shown that the pH of anoxic crucian carp blood plasma initially decrease with the onset of anoxia exposure, but stabilizes near pH 7.4 after 24 h of anoxia (15^0C) (van den Thillart and van Waarde, 1991). In comparison, blood plasma pH of the anoxic turtle continuously decrease, albeit slowly, until organismal death occurs near pH 7.0 (Herbert and Jackson, 1985a; Herbert and Jackson, 1985b; Ultsch and Jackson, 1982; Van Waarde, 1991)

The intrinsic mechanical properties of crucian carp heart exposed to a combination of anoxia and acidosis below pH 7.4 have never been examined. Does its anoxia tolerance also mean that it can tolerate acidosis? Or, is its ability to tolerate anoxia dependent on avoiding acidosis? In this thesis, these questions are examined at the level of the heart as a working tissue.

The aims of this study were to:

1. Investigate the performance of isolated spontaneous beating heart tissue preparations from crucian carp during exposure to anoxia and a stepwise decrease in pH (7.8, 7.6, 7.4, 7.2 and 7.0).
2. Measure the ability for this heart preparation to recover from anoxia in combination with three different levels of pH (7.8, 7.4 and 7.0) by comparing the rate, force and speed of contraction-relaxation before and after exposure.
3. To perform comparative measurements on the heart performance of a related, but anoxia-intolerant species.

2. Materials and methods

2.1 Experimental animals

Sixty crucian carp (*Carassius carassius* L.) of random gender, and body masses ranging between 10 and 131 g (40 ± 25 g mean S.D.) were utilized. Crucian carp were trapped in a local pond (Tjernsrud, Bærum) in Oslo early October 2007 and maintained at 6-8^{0}C for at least 3 months prior to experimentation. Fish were housed in a 370 l tank supplied with aerated and dechlorinized tap water.

Thirty-two koi carp (*Cyprinus carpio* L.) of random gender and body masses between 32 -75 g (47 ± 10 g mean S.D.) were imported from Singapore through a local supplier (Akvariefisken AS). They were slowly exposed to 6-8^{0}C from 20^{0}C over one week and then further acclimated to 6-8^{0}C for 6 weeks in a 370 l tank supplied with aerated and dechlorinized tap water.

All fish were held indoors under a 12h:12h L:D photoperiod and fed daily with commercial carp food (Tetra Pond, Tetra Melle, Germany). All experimental procedures were performed in accordance with regulations of the Norwegian Animal Research Authority.

2.2 Tissue preparation

A spontaneous contracting whole heart preparation was used to investigate the effect of extracellular anoxia and acidosis on intrinsic heart rate (f_H) and isotonic contractile properties of the crucian carp and koi carp hearts (Matikainen and Vornanen, 1992). Fish, anesthetized with buffered tricaine methanesulphonate (MM-222, Sigma, Norway) (0.4 mol l^{-1} MM-222 + 0.4 mol l^{-1} $NaHCO_3$) until opercular movement ceased, were weighed and injected with 1.0 ml kg^{-1} of heparinised saline (100 IU ml^{-1}) via the caudal blood vessels. To ensure that heparin reached the heart, the fish were returned to the anesthetic water for 1-2 min before the heart was removed.

The heart was accessed by a mid-ventral incision, gently excised and quickly rinsed in chilled saline. The heart was then placed in silicone-coated (Silastic 3481Base/81Herder, Dow Corning Corporation, USA) Petri dish that was placed on ice and contained control normoxic

physiological saline with 1 nmol l^{-1} adrenaline (see below for saline composition). Fish were subsequently killed by decapitation followed by brain destruction.

To increase the possibility for flow of saline through the contracting ventricle, the bulbous was cut off, but the atrium and sinus venosus were left intact to allow the sino-atrial-node (SA-node) to generate spontaneous APs. The ventricle was gently fixed with insect pins to the bottom of the Petri dish and two braided silk suture threads (size 5-6) were one was fastened at the outer edges of its apex and the other near the ventricular-bulbar junction. The thread attached to the apex was then fastened to a force displacement transducer (Harvard Isotonic Transducer 50-6360, Harvard Apparatus, USA) and the other thread to a fixed arm (see Fig. 3). The preparation was suspended in normoxic control physiological saline (see below for saline composition) within a water-jacketed chamber connected to a cooling system (Heto, InterMed, Denmark) that kept the temperature at 6.5 ± 0.5 ^{0}C. The transducer was attached to a manual micro-manipulator to adjust its height above the chamber, providing distance over which the ventricle could exercise by performing isotonic contractions and relaxations against a resistance made up by a spring (see Fig. 3). The system was calibrated in prior to each experiment.

The preparation was stretched carefully until no further increase in isotonic force was observed, then slightly relaxed such that it produced ~90% of its maximal contractile force. This was done to limit inter-preparation variation due to the effects of excessive cardiac stretch on spontaneous f_H and contractile tension (Cooper and Kohl, 2005; Penefsky and Hoffman, 1963). No further adjustments were made to the length of the preparation. The entire procedure lasted approximately 5 to 8 min. The experimental set-up was placed on a heavy iron table to avoid vibrations. The transducer analog signals of force were digitalized using Powerlab 4/20 and continuously recorded using Chart 5.0 software (both from AD-Instruments, UK).

In this experimental set up the contracting heart tissue preparations were pulling down the arm of the force-displacement transducer in resistance to a soft steel spring (Fig. 3). According to Hooke's law (Young and Freedman, 2004) there is a linear relationship between the extension (length units) of a spring and the force needed to cause this extension, as long as it is within the elastic limits of the spring, which was the case in this experiments.

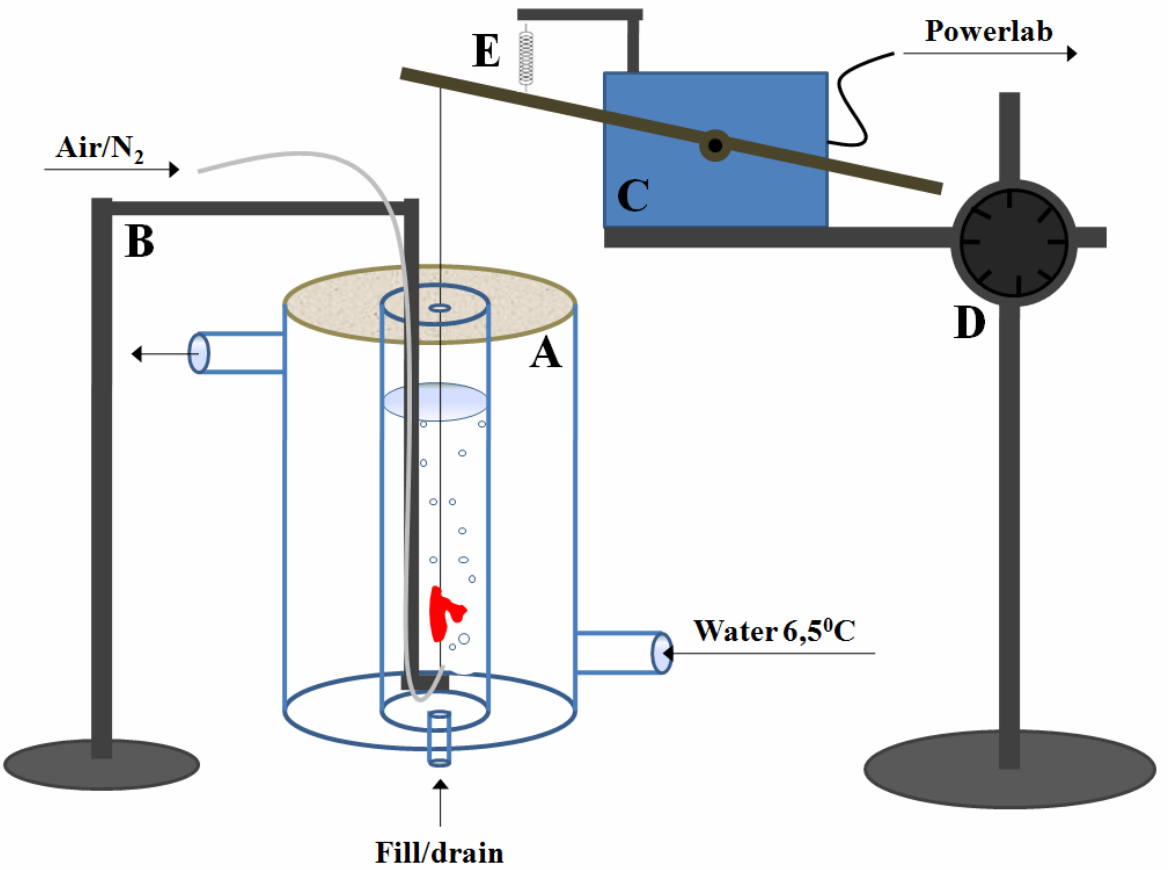

Fig. 3. Schematic representation of a heart preparation in the tissue bath. The preparation was mounted in a water-jacketed chamber (A) and fixed between a fixed arm (B) and the spring-held arm of the force-displacement transducer (C). The distance for ventricle contraction was pre-adjusted with a micro manipulator (D). The preparations performed isotonic contractions against the resistance of a soft spring (E). Contractions were digitalized by Powerlab and recorded by Chart 5.0.

2.3 Physiological saline solutions

The pH of control saline was set to 7.8, which is the natural *in vivo* extracellular pH in carp hearts at 6.5^{0}C (van den Thillart and van Waarde, 1991; Van Waarde, 1991). The physiological saline solution had the following composition (in mmol l^{-1}): NaCl 124.1, KCl 3.1, $MgSO_4 \cdot 7\ H_2O$ 0.9, $CaCl_2 \cdot 2\ H_2O$ 2.5 and *D*-Glucose 5.6. A mix between free acid and sodium salt of TES (N-[Tris(hydroxymethyl)methyl, TES free acid, 2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)amino and N-[Tris(hydroxymethyl)methyl, Sigma Aldrich, Norway) was used to maintain the appropriate pH at 6.5 ± 0.5^{0}C. The pH of the saline solution was set to either pH 7.8 (control normoxic), 7.6, 7.4, 7.2 or 7.0. The respective concentrations of TES acid and salt (in mmol l^{-1}) to obtain these pH levels were as follows: 4.83 and 2.60 for pH 7.8, 5.97 and 2.023 for pH 7.6, 7.01 and 1.50 for pH 7.4, 7.88 and 1.07 for pH 7.2 and 8.55 and

0.73 for pH 7.0. Saline solutions were refrigerated (6-8^{0}C) and glucose and adrenaline (1 nmol^{-1}) were added immediately before use. pH levels of solutions were confirmed prior to use by measurement with Metrohm 744 pH meter (Metrohm Ion Analysis, Switzerland) at 6.5 ± 1^{0}C.

2.4 Anoxia

To achieve anoxic conditions, the saline was pre-bubbled in a sealed Erlenmeyer flask (0.5 l) with 99.99% nitrogen gas (N_2) at 6-8^{0}C for at least 1.5 hour before the start of the experiment. The anoxic saline was collected with a syringe (60 ml) through a small tube and the experimental chamber injected and drained through a small opening at the bottom (see Fig. 3) as its top was tightly sealed by parafilm and tape except for a small hole that allowed for movement of the silk thread and to let the gas out. In the chamber, the anoxic solution was continuously bubbled with N_2 through PE50 tubing.

A low oxygen exposure condition was confirmed in mock experiments by measuring saline oxygen partial pressure with an oxygen probe system (WTW Oxi 340i, Wissenschaftlich-TechnischeWerkstätten GmbH, Germany) placed inside the experimental chamber during 20 min after filling. After 15 minutes, anoxic conditions were confirmed (P_{O2}< 0.3 mmHg).

2.5 Experimental protocols

All preparations were allowed to stabilize for 20 min in normoxic conditions at pH 7.8 with also served as the control period for each single heart preparation. The experimental criterion for use of suitable hearts was that f_H was beating regularly throughout the control period and that the force of contraction remained steady. Then, hearts were exposed to a series of saline solutions. Two experimental protocols were conducted.

***Experiment 1*:**

The purpose of this protocol was to test the hypothesis that crucian carp anoxic cardiac performance in anoxia is severely compromised below an extracellular pH of 7.4. Crucian carp hearts (N= 8 in all cases except crucian carp exposed to anoxia and graded acidosis where N=9) were randomly assigned to one of the following four treatments.

I) Normoxic control group:
20 min of normoxic stabilization followed by 100 min exposure to normoxic saline at pH 7.8. Saline was renewed every 20 min to replace degraded adrenaline and was continuously air-bubbled.

II) Anoxic control:
20 min of normoxic stabilization followed by 100 min exposure to anoxic saline at pH 7.8. Anoxic saline was renewed every 20 min to replace degraded adrenaline and was continuously N_2-bubbled.

III) Graded normoxic acidosis:
20 min normoxic stabilization followed by a stepwise exposure to increasingly acidotic conditions in normoxia over 100 min. pH was decreased every 20 min from 7.8 to 7.6, 7.4, 7.2 and finally 7.0. Saline was continuously air-bubbled.

IV) Graded anoxic acidosis:
20 min normoxic stabilization followed by a stepwise exposure to increasingly acidotic conditions in anoxia over 100 min. pH was decreased every 20 min from 7.8 to 7.6, 7.4, 7.2 and finally 7.0. Anoxic saline was continuously N_2-bubbled.

***Experiment 2*:**

This experiment compared the performance of crucian carp and koi carp hearts after 40 min exposure to combined anoxia and acidosis at three different pH levels. It also compared their ability to recover from treatment upon subsequent exposure to normoxic saline at pH 7.8. Crucian carp and koi carp hearts (N= 8 in all cases except crucian carp exposed to anoxia at pH 7.4 where N=11) were randomly assigned to one of the following four treatments.

I) Normoxic control:
20 min of normoxic stabilization followed by 80 min exposure to normoxic saline at pH 7.8. Saline was renewed every 20 min to replace degraded adrenaline and was continuously air-bubbled. Note: the first 80 min of normoxic control data obtained from *Experiment 1* was used as the control data

for crucian carp.

II) Anoxia at pH 7.8 followed by reoxygenation:
20 min normoxic stabilization; 40 min exposure to anoxia at pH 7.8; 40 min reoxygenation at pH 7.8. Saline was renewed every 20 min to replace degraded adrenaline and was bubbled with air during normoxia exposure and N_2 during anoxia exposure.

III) Anoxia at pH7.4 followed by reoxygenation:
20 min normoxic stabilization; 40 min exposure to anoxia at pH 7.4; 40 min reoxygenation at pH 7.8. Saline was renewed every 20 min to replace degraded adrenaline and was bubbled with air during normoxia exposure and N_2 during anoxia exposure.

IV) Anoxia at pH7.0 followed by reoxygenation:
20 min normoxic stabilization; 40 minutes exposure to anoxia at pH 7.0; 40 min reoxygenation at pH 7.8. Saline was renewed every 20 min to replace degraded adrenaline and was bubbled with air during normoxia exposure and N_2 during anoxia exposure.

2.6 Data analysis

The last 3 min of each 20 min exposure period was subsequently analyzed offline. 10 contractions from this time period were randomly selected for the measurement of the following parameters (Fig. 4):

1) Spontaneous heart rate (f_H)
2) Maximum isotonic force (F_{max})
3) Time to peak tension (TPT)
4) Time to half relaxation ($T_{1/2R}$)

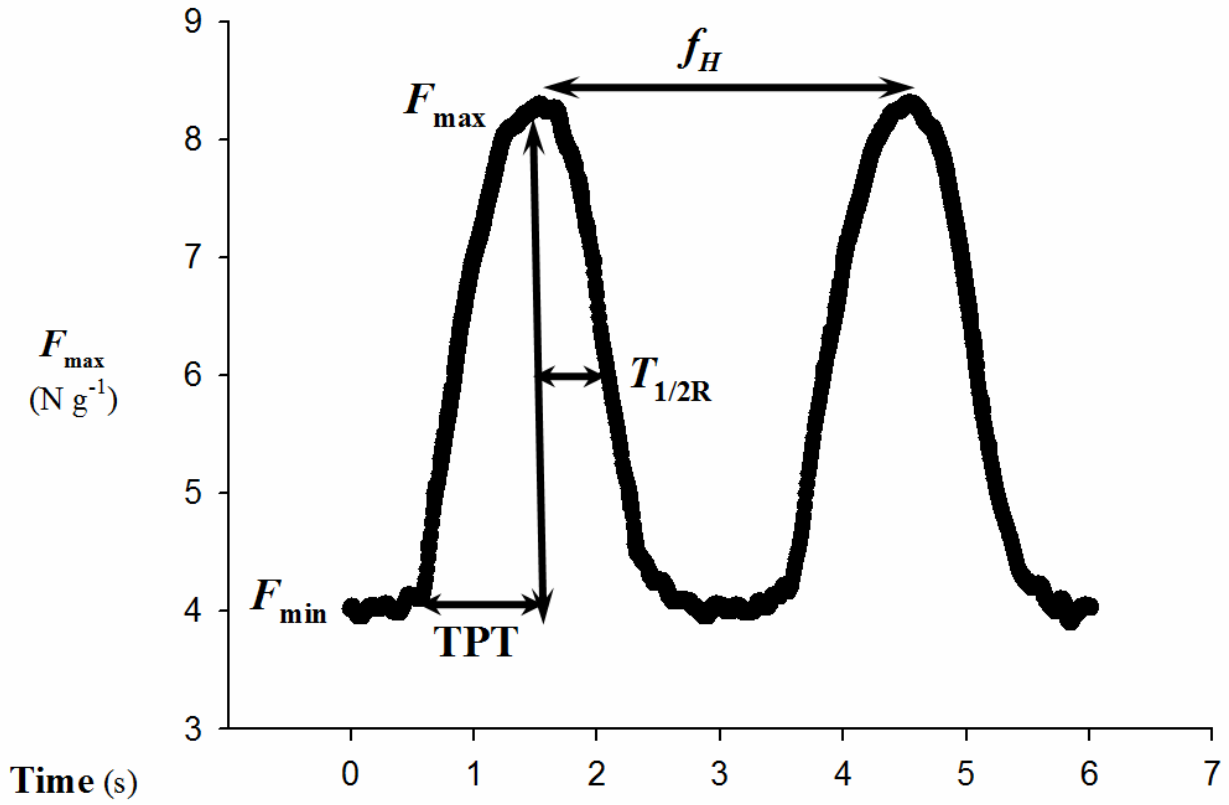

Fig. 4. Measured values of rate (f_H), maximum isotonic force (F_{max}), time to peak tension (TPT) and time to half relaxation ($T_{1/2R}$) are marked with arrows. The figure presents recording of two contractions.

f_H is the number of isotonic contractions generated per time. f_H was calculated from peak-to-peak intervals and is abbreviated as beats per minute (*bpm*). F_{max} was measured as the systolic peak amplitude of the contraction relative to the diastolic baseline ($F_{max} - F_{min}$) and is presented as Newton per gram tissue (N g^{-1}). TPT was measured as the time interval between F_{min} and F_{max} and is presented in seconds. $T_{1/2R}$ was calculated by the time interval in seconds between F_{max} and half relaxation of tension (i,e. $F_{max} - ((F_{max}\text{-}F_{min})/2)$. As previously described (Matikainen and Vornanen, 1992), the product of f_H and F_{max} was used to estimate the cardiac output and is termed 'pumping capacity' (PC) in N g^{-1} min^{-1}.

2.7 Statistical analysis

Data presented as mean ± S.E.M. All statistical analysis were conducted with SigmaStat 3.0 (Aspire Software International, USA). A one-way repeated measures analysis of variance (ANOVA) was used to determine statistically significant variations in measured variables

over time (statistical differences indicated by dissimilar numbers in Figs. 5 – 9, 12 and 13) and a one-way ANOVA was used to determine statistically significant differences between treatment groups at a specific exposure time (statistical differences indicated by dissimilar letters in Figs. 5 – 9, 12 and 13). For *Experiment 2*, a one-way ANOVA was used to determine statistically significant differences in normalized data between treatment groups at 40 min of exposure and 40 min of recovery (statistical differences indicated by dissimilar letters in Figs. 12 and 13). In all instances, $P < 0.05$ was used as the level for significance and multiple comparisons were performed using Student Newman-Keuls post tests.

3 Results

3.1 *Experiment 1*

3.1.1 Stability of the heart preparation

Normoxic control hearts (NC) remained viable throughout the duration of the experiment. No significant changes in f_H (Fig. 5), F_{max} (Fig. 6) or $T_{1/2R}$ (Fig. 9) occurred between 0 and 100 min. PC was decreased slightly by 16.9 ± 7.4% from control (i.e.., time = 0 min) after 60 min (Fig. 7), and TPT was reduced by 6.6 ± 2.1% from control after 40 min (Fig. 8). However, no significant variation in PC or TPT occurred between 20 and 100 min of exposure.

3.1.2 Effect of graded acidosis in normoxia

Heart preparations exposed to graded acidosis during normoxia (NA) performed at a stable level throughout the 100 min exposure in regards to f_H (Fig. 5), PC (Fig. 7) and TPT (Fig. 8). F_{max} increased slightly by 12.5 ± 5.9% after 20 min (i.e., at pH 7.8), but no further change occurred with exposure to more severe acidosis (Fig. 6). $T_{1/2R}$ was only slightly slower than normoxic control (by 9.1 ± 4.7%) when exposed to pH 7.0 (Fig. 9). Thus, the crucian carp heart preparations were tolerant to extracellular acidosis when oxygen was available.

3.1.3 Effect of anoxia

Anoxia exposure alone caused immediate reductions in f_H, F_{max} and PC. Specifically, f_H, F_{max} and PC were reduced by 15.9 ± 5.6%, 24.7 ± 8.3% and 38.7 ± 6.2%, respectively, after 20 min of anoxia exposure (Fig. 5, 6 and 7). By 40 min, f_H was reduced by 29.7 ±7.1% (Fig. 5). However, f_H, F_{max} and PC subsequently stabilized beyond 20 – 60 min of exposure. Simultaneously, TPT and $T_{1/2R}$ increased by 13.6 ± 6.6% and 87.9 ± 31.4%, respectively, by 60 min. However, like for f_H, F_{max} and PC, following this initial change, no further significant alteration of TPT or $T_{1/2R}$ occurred. Thus, cardiac performance of the heart preparations decreased to a new “steady-state” in response to anoxia exposure.

3.1.4 Effect of combined anoxia and graded acidosis

f_H of the preparations exposed to combined anoxia and graded acidosis (AA) decreased similarly to the AC group during the first 60 min of exposure even though extracellular pH was decreased from 7.8 to 7.6 at 20 min and subsequently from 7.6 to 7.4 at 40 min (Fig. 5). Thus, at 60 min of exposure (i.e., pH 7.4 for the AA group), the magnitude of reduction in f_H from control (i.e., t = 0 min) was nearly identical between the AC group (decreased by 39 ± 5.3%) and AA group (decreased by 38.7 ± 7.8%). However, in contrast to the AC group, in which f_H subsequently stabilized after 40 min of anoxia exposure, f_H of AA hearts further decreased upon exposure to pH 7.2 (i.e., t = 80 min). As a result, f_H was 4.1 and 4.8 bpm slower than comparable AC hearts at 80 and 100 min of exposure when extracellular pH was 7.2 and 7.0, respectively.

Similar to the f_H response, the pattern of change of TPT and $T_{1/2R}$ of AA hearts was quantitatively similar to that exhibited by the AC hearts during the first 60 min of exposure even though the bath pH was made increasingly more acidotic (Fig. 8 and 9). However, below pH 7.4, TPT and $T_{1/2R}$ of the AA hearts continued to slow, whereas TPT and $T_{1/2R}$ of the AA hearts had stabilized. Consequently TPT of AA hearts was 0.3 and 0.5 s longer, and $T_{1/2R}$ was 0.6 and 0.9 s longer than comparable AC hearts when pH was 7.2 (i.e., t = 80 min) and 7.0 (i.e., t = 100 min), respectively. Thus crucian carp heart preparations exposed to a combination of anoxia and an extracellular pH below 7.4 did not beat as frequently or contract and relax as quickly as anoxic hearts at higher extracellular pH (Fig. 11).

Unlike the response observed for f_H, TPT and $T_{1/2R}$, the pattern of change of F_{max} and PC did not differ between AC and AA hearts (Fig. 6 and 7). Similar to the AC hearts, F_{max} of AA hearts initially decreased by 39.3 ± 6.7% after 20 min of exposure, but did not further decrease despite graded exposure to pH 7.6, 7.4, 7.2 and finally 7.0. Similarly, PC of AA hearts was decreased by 32.5 ± 14.6% after 20 min of anoxia (when pH was still 7.8) and by 69.8 ± 5.7% after 60 min (when pH was 7.4). No further changes in F_{max} and PC occurred after these initial alterations. Thus, despite the negative effect of combined anoxia and acidosis below pH 7.4 on f_H and contraction kinetics, crucian carp hearts exposed to these conditions were able to still produce a contraction force and have a PC equal to anoxia hearts at pH 7.8.

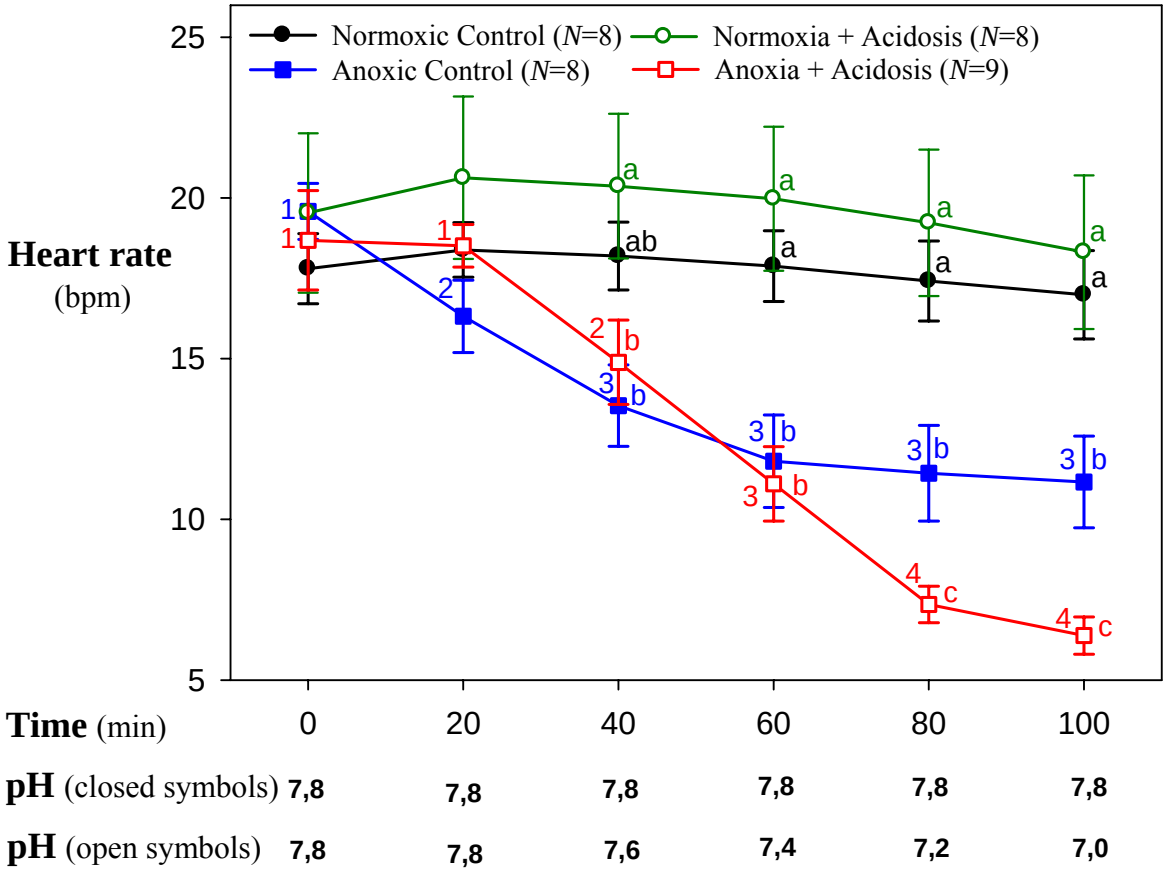

Fig. 5. Spontaneous f_H of the four experimental groups of crucian carp heart preparations. Circles = preparations maintained in normoxia; Squares = anoxia-exposed preparations (except time = 0); Closed symbols = pH maintained at 7.8; Open symbols = preparations exposed to graded acidosis. Data are presented as means ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group are indicated by dissimilar numbers. Statistically significant differences ($P < 0.05$) between experimental groups at a specific time are indicated by dissimilar letters.

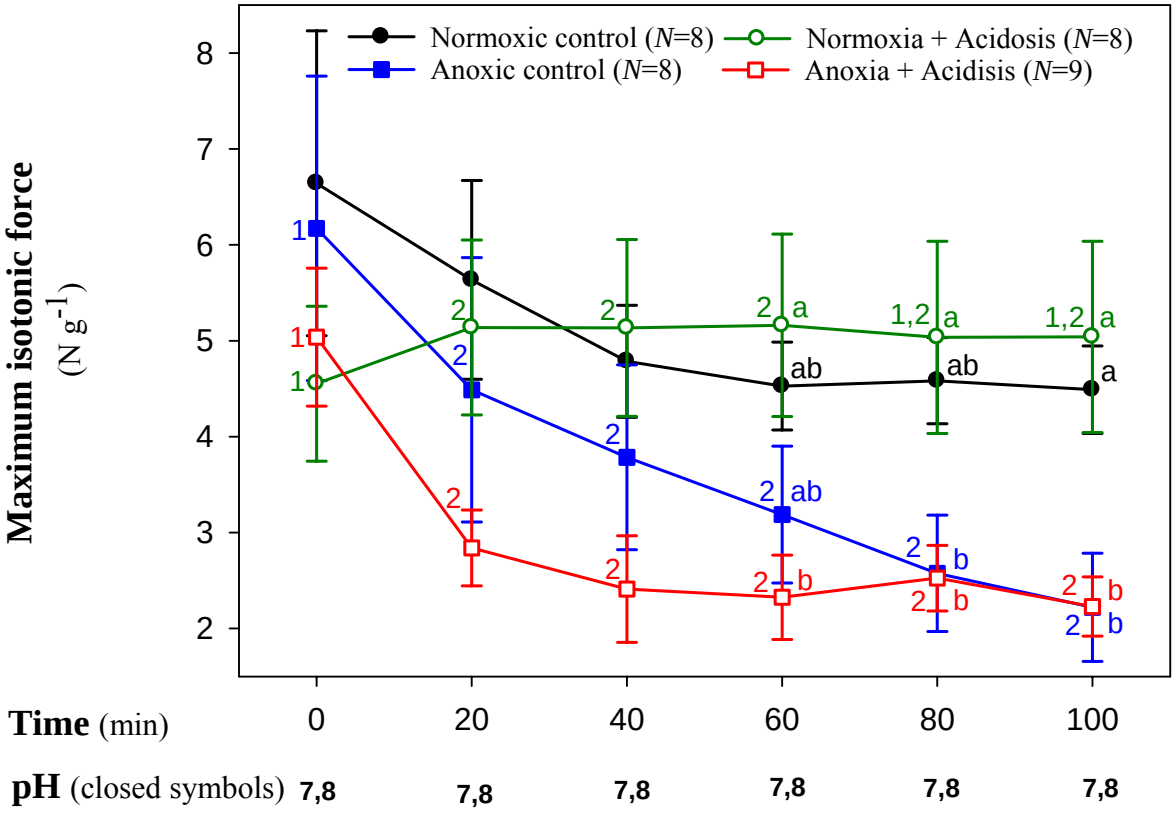

Fig. 6. F_{max} of the four experimental groups of crucian carp heart preparations. Circles = preparations maintained in normoxia; Squares = anoxia-exposed preparations (except time = 0); Closed symbols = pH maintained at 7.8; Open symbols = preparations exposed to graded acidosis. Data are presented as means ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group are indicated by dissimilar numbers. Statistically significant differences ($P < 0.05$) between experimental groups at a specific time are indicated by dissimilar letters.

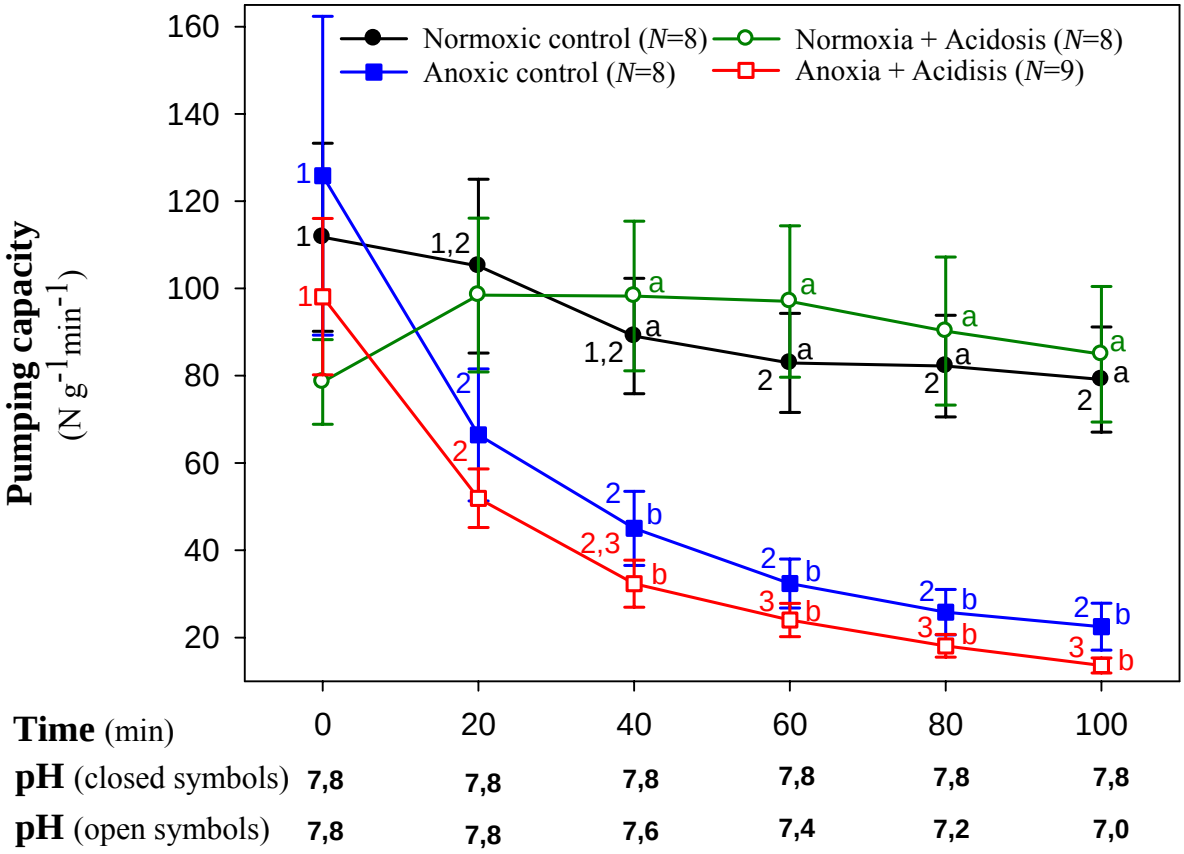

Fig. 7. PC of the four experimental groups of crucian carp heart preparations. Circles = preparations maintained in normoxia; Squares = anoxia-exposed preparations (except time = 0); Closed symbols = pH maintained at 7.8; Open symbols = preparations exposed to graded acidosis. Data are presented as means ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group are indicated by dissimilar numbers. Statistically significant differences ($P < 0.05$) between experimental groups at a specific time are indicated by dissimilar letters.

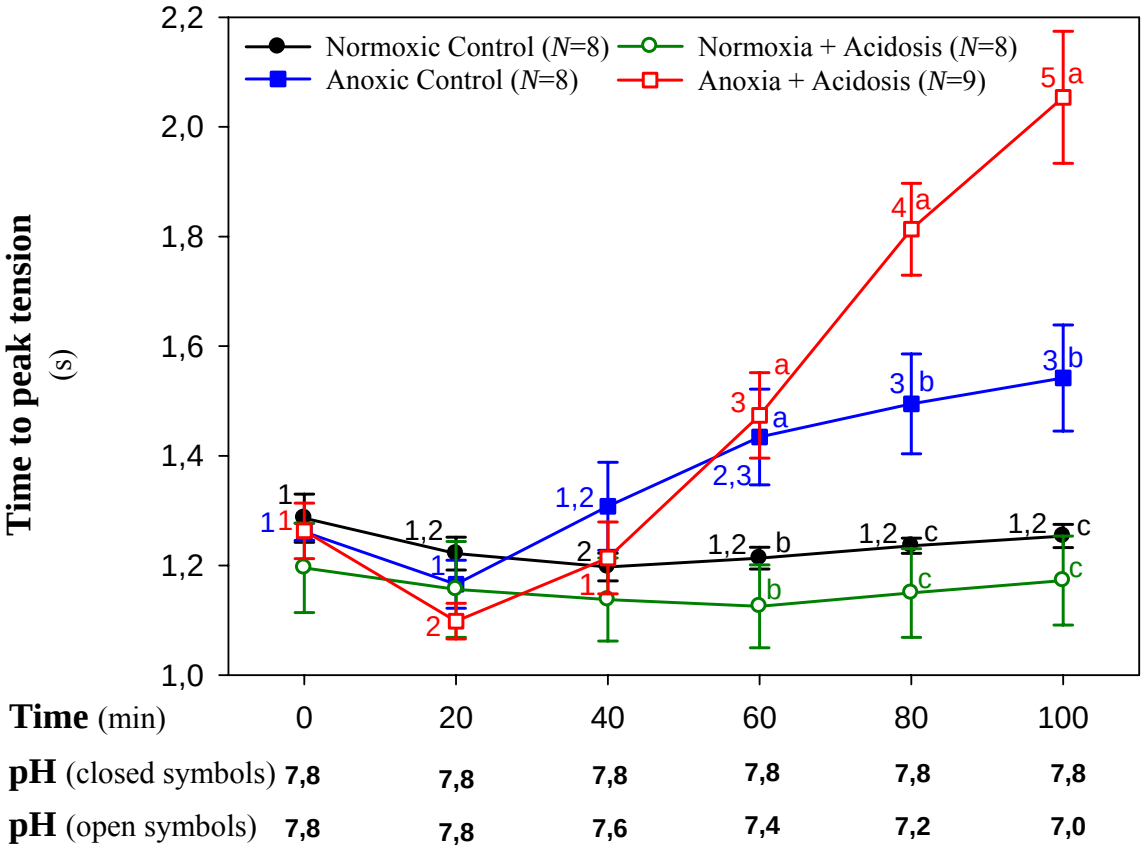

Fig. 8. TPT of the four experimental groups of crucian carp heart preparations. Circles = preparations maintained in normoxia; Squares = anoxia-exposed preparations (except time = 0); Closed symbols = pH maintained at 7.8; Open symbols = preparations exposed to graded acidosis. Data are presented as means ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group are indicated by dissimilar numbers. Statistically significant differences ($P < 0.05$) between experimental groups at a specific time are indicated by dissimilar letters.

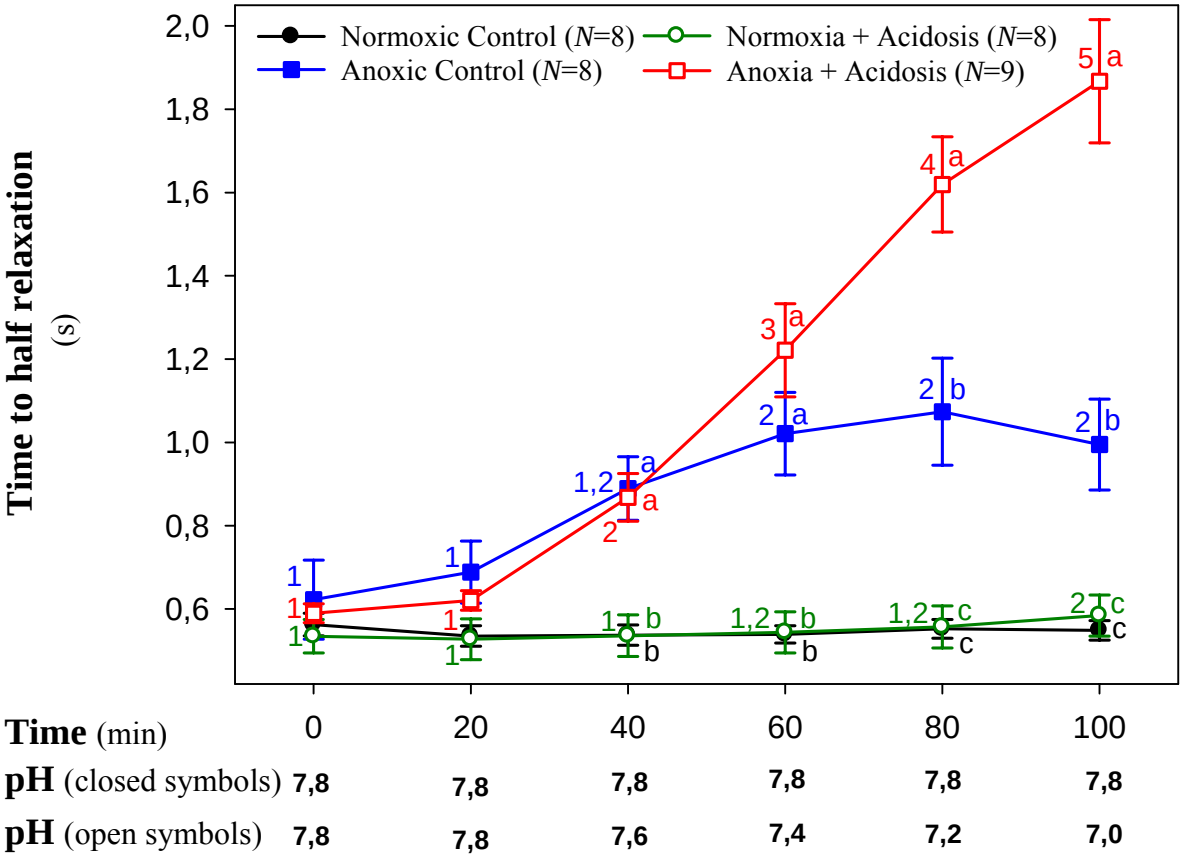

Fig. 9. $T_{1/2R}$ of the four experimental groups of crucian carp heart preparations. Circles = preparations maintained in normoxia; Squares = anoxia-exposed preparations (except time = 0); Closed symbols = pH maintained at 7.8; Open symbols = preparations exposed to graded acidosis. Data are presented as means ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group are indicated by dissimilar numbers. Statistically significant differences ($P < 0.05$) between experimental groups at a specific time are indicated by dissimilar letters.

3.1.4 Summary

A schematic of the general responses of isolated spontaneous contracting crucian carp heart preparations to the four different exposures of *Experiment 1* is presented in Fig. 10. The following trends were observed.

NC: Normoxic control hearts kept at pH 7.8 for 100 min exhibited stable performance in all manners except for relatively minor alterations of PC and TPT.
NA: Normoxic hearts exposed to graded acidosis exhibited stable performance in all manners except for F_{max} and $T_{1/2R}$, in which only relatively small changes occurred.
AC: Anoxia exposure at pH 7.8 initially caused f_H, F_{max} and PC to decrease, while TPT and $T_{1/2R}$ increased. However, these early changes were followed by a stabilization of cardiac performance.
AA: Anoxic hearts exposed to combined anoxia and graded acidosis exhibited changes in $f_{H,}$ TPT and $T_{1/2R}$ that were all similar to the responses of the AC group until pH was reduced below 7.4. Thus, when exposed to more severe extracellular acidosis (i.e., pH 7.2 and 7.0), $f_{H,}$ TPT and $T_{1/2R}$ were all significantly slower than in comparable AC hearts. No statistically significant difference in F_{max} and PC were seen between AC and AA hearts at any pH tested.

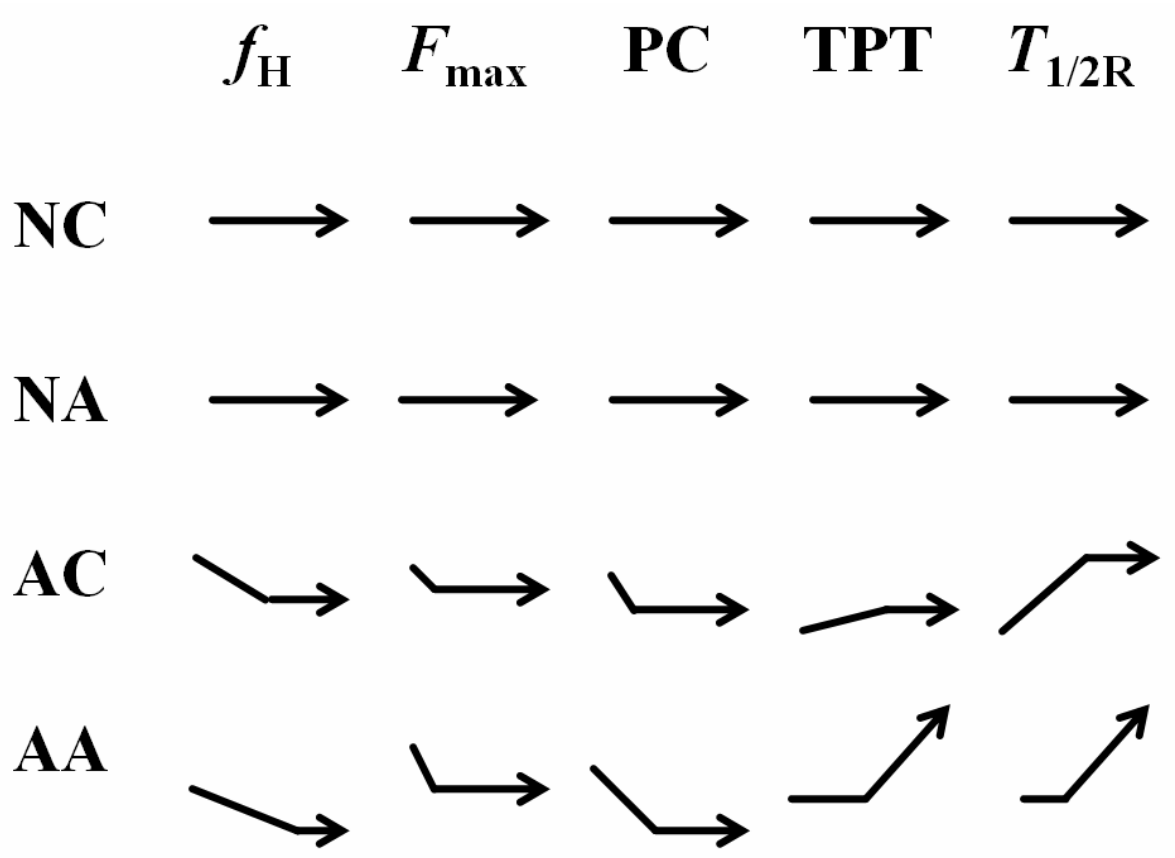

Fig. 10. Schematic representation of the general responses of isolated spontaneous contracting crucian carp heart preparations to the four different exposures of *Experiment 1*. Arrows indicate: (→) = stable over 100 min: (↘ and ↗) = an initial decrease or increase followed by stabilization compared to time zero: and (↗) = a shift to continued increase. The location of the bend and length of line before the bend indicates the relative magnitude and the time at which the change in cardiac performance occurred.

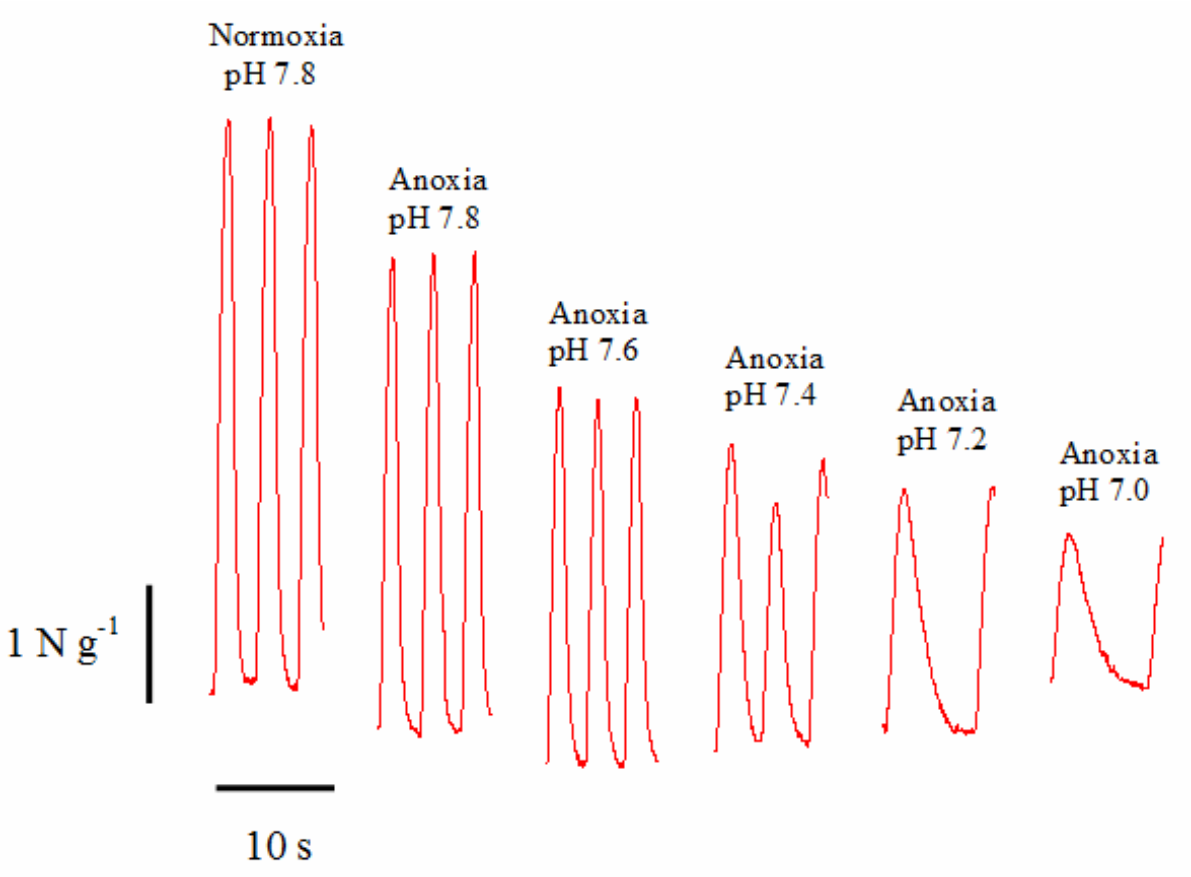

Fig. 11. Representative recordings of cardiac contractions performed by an isolated crucian carp heart preparation exposed to anoxia and graded acidosis. At each pH, contractions during a 10 s period are displayed.

3.2 *Experiment 2*

3.2.1 Stability of the heart preparations

The total duration of *Experiment 2* was 80 min. Therefore, rather than unnecessarily sacrificing additional fish, the first 80 min of NC crucian carp data from *Experiment 1* was utilized as the control normoxic data for *Experiment 2*. As described above (section 3.1.1), the crucian carp heart preparation was viable throughout the 80 min experiment as it did not show any significant alteration of f_H, $F_{max,}$ PC and $T_{1/2R}$. TPT was reduced from control by 6.6 ± 2.1% at 40 min, but it returned to the control level by 80 min (Fig. 13A).

Like for crucian carp hearts, normoxic koi carp heart preparations (NC) also remained viable for the duration of the experiment. No statistically significant differences in f_H or PC occurred between 0 and 80 min (Fig. 12B and F). F_{max}, TPT and $T_{1/2R}$ did decrease from control levels after 80 min, but the magnitude of change was small, being 6.3 ± 2.4% for F_{max}, 7.5 ± 2.5% for TPT and 7.6 ± 1.4% for $T_{1/2R}$ (Fig. Figs. 12D and 13B and D).

3.2.2 Comparison of the effect of anoxia at extracellular pH 7.8, 7.4 and 7.0 on crucian carp and koi carp cardiac performance

Crucian carp and koi carp responded similarly to a 40 min anoxia exposure regardless if the extracellular pH was pH 7.8, 7.4 or 7.0 (Table 1; Figs 12 and 13).

With anoxia exposure at pH 7.8, f_H of crucian carp and koi carp was decreased by ~27% and PC by ~57% (Figs. 12A and E; Table 1). No significant differences from comparable NC hearts occurred for F_{max} or TPT in either species (Fig. 12C and D; Fig. 13A and B), but F_{max} was reduced by ~37% from the pre-exposure control level in both crucian carp and koi carp (Table 1). $T_{1/2R}$ was increased from the pre-exposure control level by 76.0 ± 18.4% for crucian carp (Fig. 13C) and similarly by 82.4 ± 15.0 % for koi carp (Fig. 13D). However, the slower $T_{1/2R}$ of koi carp was not statistically significantly different from comparable NC hearts at 40 min.

With anoxia exposure at pH 7.4, crucian carp f_H was decreased by 57.2 ± 6.0% (Fig. 12A) and koi carp f_H by 44.5 ± 5.2% (Fig. 12B). Crucian carp PC was reduced by 78.4 ± 2.9% (Fig. 12E) and koi carp PC by 73.0 ± 3.0% (Fig. 12F). F_{max} was decreased by 57.6 ± 6.1% in

crucian carp hearts and by 46.0 ± 9.0% in koi carp hearts (Fig. 12C and D), but no significant change from comparable NC hearts occurred for F_{max} of koi carp (Table 1). Likewise, the slowed TPT and $T_{1/2R}$ of koi carp hearts exposed to anoxia at pH 7.4 were similar to that displayed by crucian carp despite the fact that the magnitudes of change were not significantly different from comparable NC hearts (Fig. 13; Table 1). Nevertheless, the decreased contraction kinetics of koi carp hearts at 40 min was statistically slower than pre-exposure control (Table 1).

With anoxia exposure at pH 7.0, crucian carp f_H was reduced by 60.1 ± 4.3% (Fig. 12A) and PC by 72.1 ± 4.4% (Fig. 12E). For koi carp, the reduction in f_H and PC were again similar to the crucian carp, being 46.0 ± 10.8% (Fig. 12B) and 84.2 ± 2.4% (Fig. 12F), respectively. F_{max} was decreased 57.6 ± 6.1% for crucian carp (Fig. 12C) and similarly by 59.2 ± 10.8% (Fig. 12D) for koi carp. TPT increased by 32.8 ± 4.9% in crucian carp (Fig. 13A) and 50.5 ± 20.1% in koi carp (Fig. 13B). $T_{1/2R}$ increased by 204.8 ± 20.1% and 206.4 ± 31.7% in crucian carp and koi carp hearts, respectively (Fig. 13C and D).

In general, exposure to anoxia at more severe extracellular pH resulted in greater negative alterations of cardiac performance. This phenomenon was most clearly pronounced for PC of koi carp hearts (Fig. 12F). A similar trend existed for f_H, F_{max}, TPT and $T_{1/2R}$ (Fig. 12B and D; Fig. 13B and D). For crucian carp, the alterations in f_H, F_{max}, PC, TPT and $T_{1/2R}$ with anoxia exposure at pH 7.4 were all significantly more severe than during anoxia exposure at pH 7.8 (Fig. 12A, C and E; Fig. 13A and C). Interestingly, no further statistically significant reductions in cardiac performance beyond that observed at pH 7.4 occurred in crucian carp hearts exposed to anoxia at pH 7.0 (Figs. 12 and 13). In fact, F_{max} of crucian carp hearts exposed to anoxia at pH 7.0 was actually less disrupted than that of hearts exposed to anoxia at pH 7.4 (Fig. 12C).

3.2.3 Comparison of the ability of crucian carp and koi carp hearts to recover from anoxia exposure at extracellular pH 7.8, 7.4 and 7.0

Despite the many similar responses of crucian carp and koi carp hearts to 40 min of anoxia exposure regardless of the extracellular pH, the hearts of the two species differed substantially in their ability to recover cardiac performance upon subsequent reoxygenation. Hearts of both species recovered f_H to the comparable NC levels upon reoxygenation regardless of the pH they were exposed to during anoxia (Fig. 12A and B). Crucian carp hearts were also able to

recover F_{max} and PC, with the exception of F_{max} of hearts exposed to anoxia at pH 7.4 (Fig. 12C and E). In comparison, F_{max} or PC of any group of anoxia-exposed koi carp hearts did not recover to control normoxic levels upon reoxygenation. Rather, these parameters remained approximately 25 - 50% lower than comparable NC hearts at 40 min of reoxygenation (Fig. 12D and F). Thus, crucian carp hearts were superior to koi carp hearts in their ability to recover contraction force and PC following an anoxic insult.

Interestingly, an opposite pattern of recovery ability was observed for contraction kinetics (Fig. 13). Here, TPT and $T_{1/2R}$ of anoxia-exposed crucian carp hearts did not recover to comparable NC levels after 40 min of reoxygenation, whereas TPT and $T_{1/2R}$ of koi carp did recover successfully. However, this pattern likely reflects the general increase in f_H and decrease in F_{max} of koi carp hearts by the conclusion of the experiment, which inherently quickened contraction kinetics (see Fig. 12A and B and Fig. 14)

Table 1. ***Comparison of the effect of 40 min anoxia exposure at different extracellular pH on the cardiac performance of crucian carp and koi carp heart preparations.***

pH	Parameter	% change from pre-exposure control level			
		Crucian carp		Koi carp	
7.8	f_H	-30.7 ± 10.6	*†	-24.9 ± 6.9	*†
	F_{max}	-36.0 ± 7.5	†	-38.7 ± 7.1	†
	PC	-58.7 ± 5.4	*†	-55.9 ± 5.2	*†
	TPT	+6.2 ± 4.5		+11.9 ± 5.0	
	$T_{1/2R}$	+76.0 ± 18.4	*†	+82.4 ± 15.0	†
7.4	f_H	-57.2 ± 6.0	*†	-44.5 ± 5.2	*†
	F_{max}	-57.6 ± 6.1	*†	-46.0 ± 9.0	†
	PC	-78.4 ± 2.9	*†	-73.0 ± 3.0	*†
	TPT	+46.1 ± 9.6	*†	+28.3 ± 5.6	†
	$T_{1/2R}$	+154.6 ± 24.7	*†	+132.4 ± 15.4	†
7.0	f_H	-60.1 ± 4.3	*†	-46.0 ± 10.8	*†
	F_{max}	-31.3 ± 4.9	†	-59.2 ± 10.8	*†
	PC	-72.1 ± 4.4	*†	-84.2 ± 2.4	*†
	TPT	+32.8 ± 4.9	*†	+50.5 ± 20.1	*†
	$T_{1/2R}$	+204.8 ± 20.1	*†	+206.4 ± 31.7	*†

An asterisk indicates a statistically significant difference ($P < 0.05$) from comparable NC hearts for each species. A cross indicates a statistically significant difference ($P < 0.05$) from the pre-exposure control level as determined with a one-way RM-ANOVA on absolute values. Values are means ± S.E.M. N= 8 for all groups except for crucian carp at pH 7.4 were N = 11.

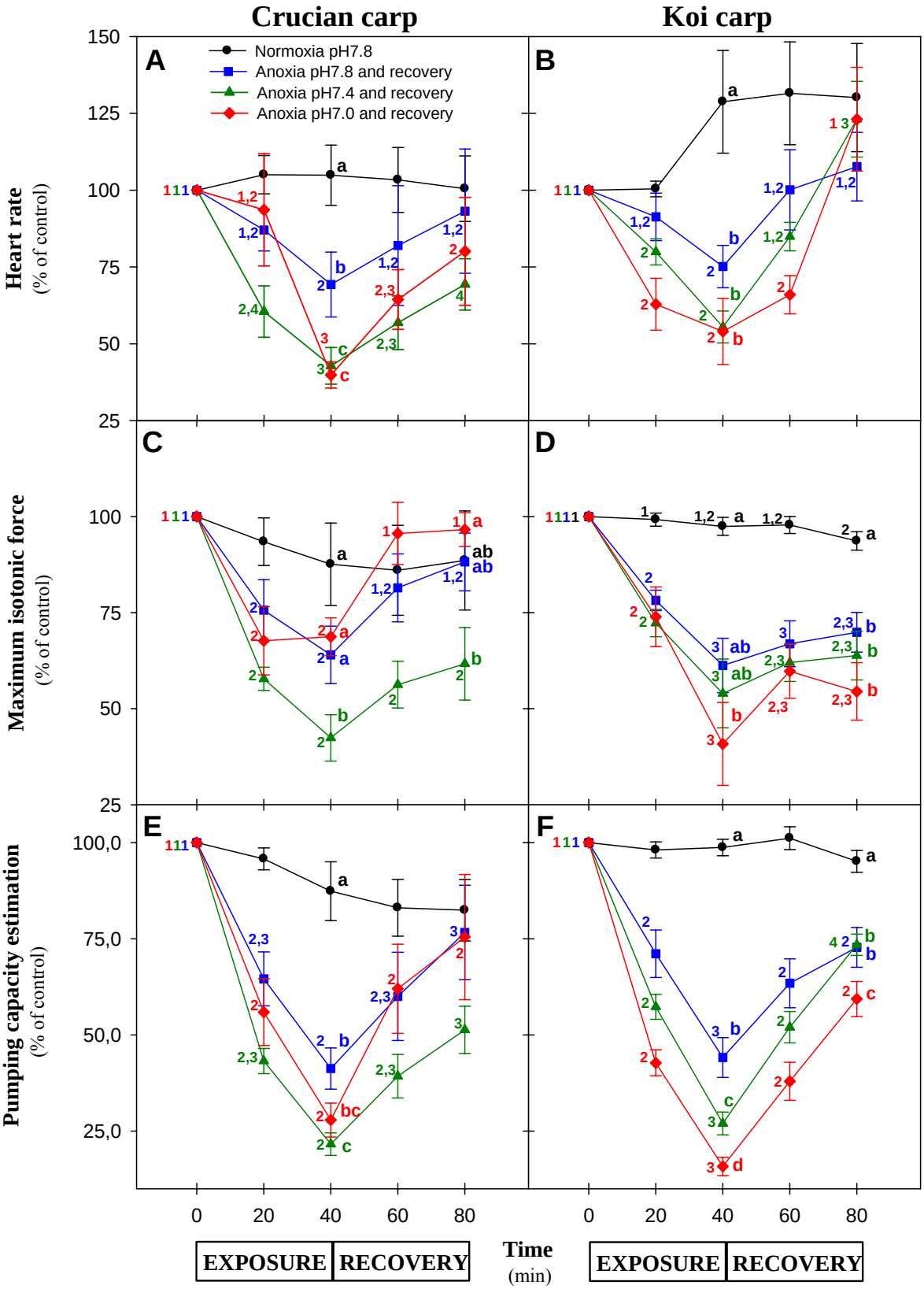

Fig. 12. Comparison of the effect of 40 min anoxia exposure at different pH and subsequent 40 min reoxygenation at pH 7.8 on f_H (A and B), F_{max} (C and D) and PC (E and F) of crucian carp and koi carp spontaneously contracting heart preparations. Data are presented as normalized values in mean ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group as determined by a one-way RM ANOVA on absolute values are indicated by dissimilar numbers. Statistically significant differences between exposure groups at 40 and 80 min, as performed with a one-way ANOVA on normalized data, are indicated by dissimilar

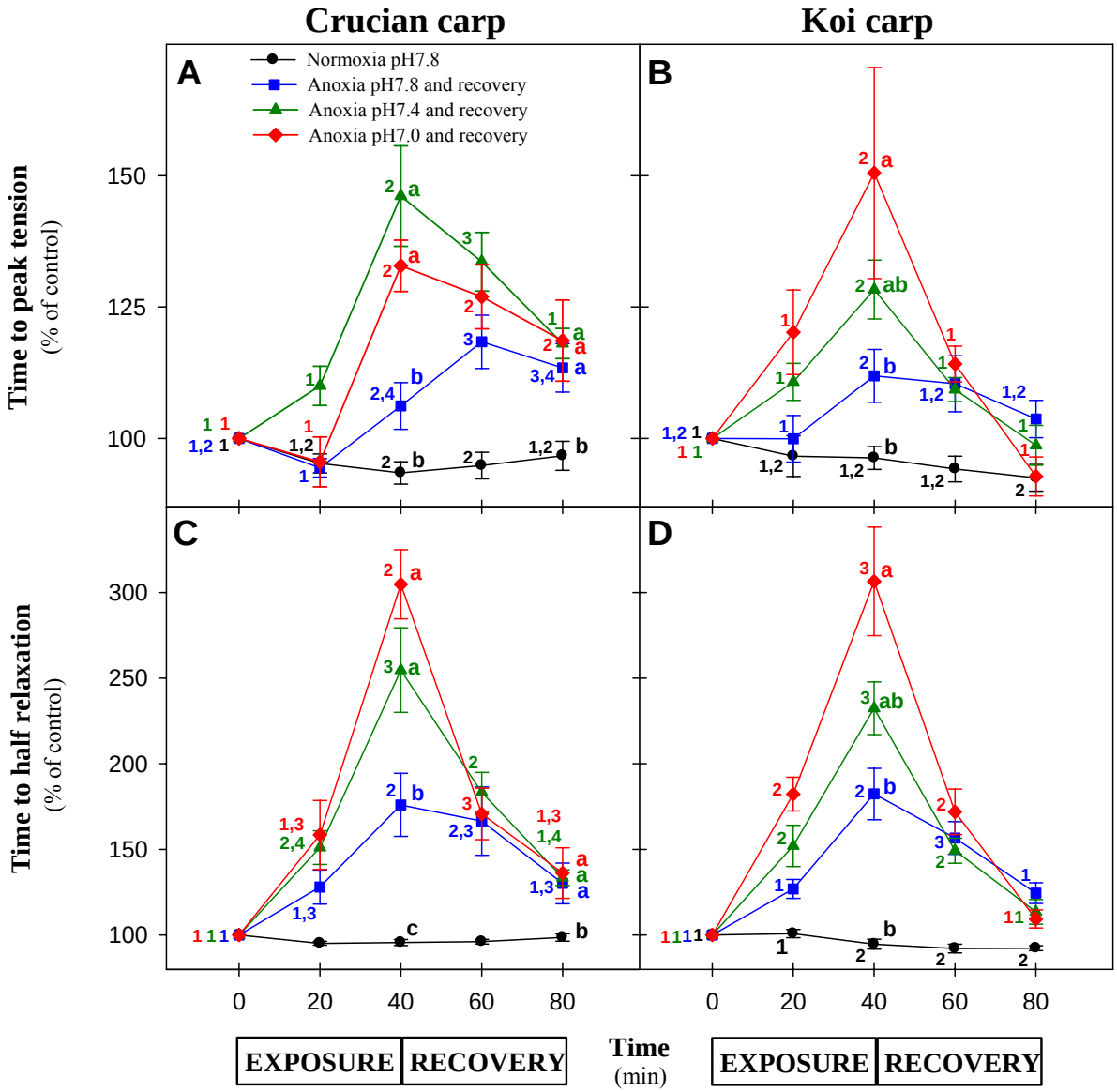

Fig. 13. Comparison of the effect of 40 min anoxia exposure at different pH and subsequent 40 min reoxygenation at pH 7.8 on TPT (A and B) and $T_{1/2R}$ (C and D) of crucian carp and koi carp spontaneously contracting heart preparations. Data are presented as normalized values in mean ± SEM. Statistically significant changes ($P < 0.05$) over time for each experimental group as determined by a one-way RM ANOVA on absolute values are indicated by dissimilar numbers. Statistically significant differences between exposure groups at 40 and 80 min, as performed with a one-way ANOVA on normalized data, are indicated by dissimilar letters ($P < 0.05$).

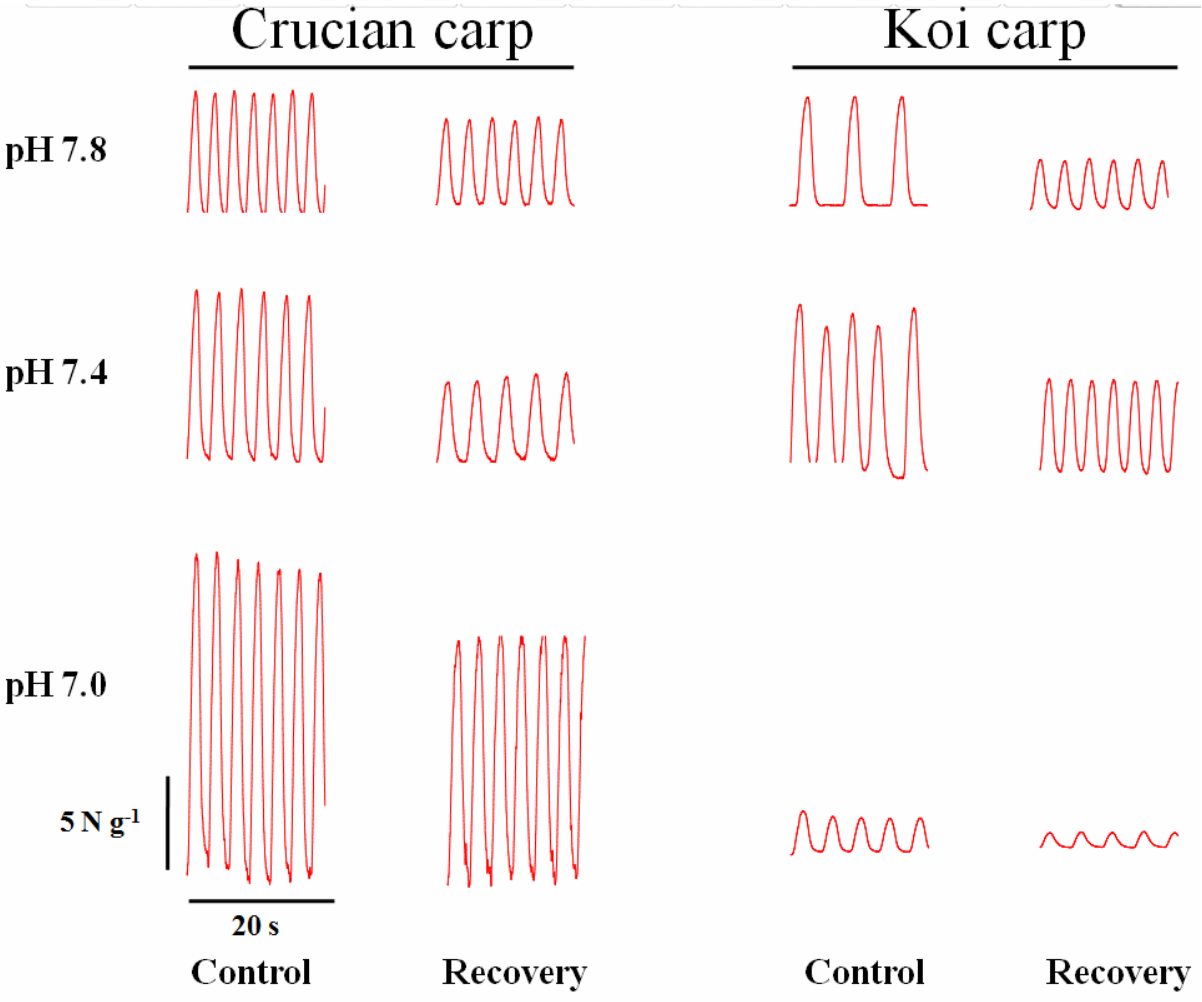

Fig. 14. Representative contractions of crucian carp and koi carp heart preparations before and after treatment of 40 min anoxia at pH 7.8, pH 7.4 or pH 7.0. Each example shows 20 s from the normoxic control period and 20 s from the conclusion of the recovery period.

4 Discussion

4.1 Critique of methods

The crucian carp utilized in these experiments were captured from a natural pond during October 2007. Fish size was uneven, ranging between 10 and 130 g (40 ± 25 g mean S.D.), which may have contributed to the inter-preparation variation. Still, the apparent changes seen were often statistically significant, suggesting that inter-preparation differences were not unduly high.

The natural *in vivo* extracellular pH of crucian carp at 6.5^0C was calculated from the measurement of blood plasma pH at 15^0C (van den Thillart and van Waarde, 1991) using alpha-stat correction (0.016 pH units $deg.^{-1}$) for temperature differences (Overgaard et al., 2004).

During installation of the heart preparation in the experimental set-up, the myocardium was for a short time (about 5-10 s) exposed to air. This could have induced some mechanisms of preconditioning for the subsequent anoxic treatment (Gamperl et al., 2001) resulting in enhanced cardiac performance. However, care was taken to handle each heart in the same manner, so any preconditioning effect should apply to all hearts.

Three mock experiments were performed to confirm the flow of saline through the atrium and ventricle during cardiac contraction. When hearts were pinned to the bottom of a silicone-coated Petri dish and 5 µl of dye (food color) was introduced close to the atria the dye was observed to be sucked in to the atria and pumped out of the ventricle. Moreover, the fact that modification of the saline solution had significant effect on heart performance indicates that the cardiac tissue experienced changing extracellular conditions.

In nature, the crucian carp experience anoxia during the winter months at cold water temperatures down to 4^0C at the bottom of ice-covered ponds (Holopainen and Hyvarinen, 1985). Experiments were conducted at a cold temperature in an attempt to mimic the natural temperature during winter anoxia, but acclimation temperature was limited by the temperature of the tap water supplying the fish tanks (6-8^0C). The experimental temperature (6.5 ± 0.5^0C) was within this range.

To make useful extrapolation to the *in vivo* situation, the spontaneously contracting heart preparations should be stable for the duration of the experimental protocol and contract at

rates comparable to *in vivo* intrinsic f_H. This was indeed the case. NC crucian carp and koi hearts performed at a stable level for the entire duration of the experimental protocols, displaying only minor alterations of the measured parameters (Fig. 5-9, 12 and 13). Care was taken to ensure similar test conditions during each experiment except for the variations of treatment. Further, f_H of crucian carp hearts under normoxia (17.8 ± 1.2 bpm) was identical to the previously reported *in vivo* intrinsic f_H of ~17 bpm (Stecyk et al., 2004). Likewise, for koi carp hearts, spontaneous f_H during normoxia (18.0 ± 1.1 bpm) falls within the range of in vivo intrinsic f_H 8.2 ± 1.1 bpm at 5^0C and 19.4 ± 1.3 bpm at 10^0C (Stecyk and Farrell, 2006). This similarity of spontaneous f_H in the present study and *in vivo* intrinsic f_H indicates that the heart preparations were performing at a physiology relevant level.

4.2 *Experiment 1*

Four groups of isolated spontaneous contracting crucian carp heart preparations were used to investigate cardiac response to acidosis, anoxia and anoxia combined with graded acidosis.

4.2.1 Heart response to acidosis during normoxia

With oxygen available, the crucian carp hearts exposed to graded acidosis (the NA group) performed similar to NC hearts (Fig. 5-9). This finding indicates that in the presence of oxygen, decreased extracellular pH did not negatively affect pacemaker cells firing frequency or the contractile properties of cardiomyocytes. Extracellular acidosis can occur in the body by two means: (1) Respiratory acidosis (elevated blood CO_2 concentration) and (2) metabolic acidosis (lowered HCO^{3-} and/or increased production of H^+), and each type has different effects on cardiac contractility (Williamson et al., 1976). For example, an increase in CO_2 concentration decreases intracellular pH more than lowered bicarbonate due to the faster diffusion of CO_2 tan bicarbonate across the sarcolemma (Driedzic and Gesser, 1994). The acidotic conditions utilized in these experiments were achieved by utilizing the artificial buffer (TES) and are thus comparable to a metabolic acidosis.

Generally, acidosis is known to negatively affect cardiac performance by reducing f_H (Satoh and Hashimoto, 1983; Severi et al., 2002) and contractility (Driedzic and Gesser, 1994; Gesser and Poupa, 1983). For instance, perfused painted turtle hearts (*Chrysemys picta bellii* L.) exposed to a lactic acidosis of pH 7.0 for 4 h in normoxia, reduced cardiac output by 16% after 1.5 h (Wasser et al., 1990), and hearts of red-eared slider turtles (*Trachemis scripta*

elegans L.) exposed to similarly graded acidosis by CO_2 elevation and by increased concentration of lactic acid for 30 min between pH 7.7 to 6.9 responded by decrease in twitch force by 20% (Kalinin and Gesser, 2002). Likewise, rainbow trout (*Salmo gardneri* L.) hearts exposed to metabolic acidosis at pH 7.4 in 10-15 min decreased f_H by 19% and cardiac output by 17%, respectively (Farrell et al., 1988). Further, contractile force of rainbow trout hearts have been shown to decrease by ~30% after 5 min and continue to decrease to 50% of control after 30 min of hypercapnic acidosis (pH 7.0) at 22^0C (Gesser and Jørgensen, 1982).

However, some animal hearts are to some extent tolerant to acidosis. For instance, the flounder (*Pleuronectes flesus* L.) heart contractility exhibits a biphasic response to graded, normoxic hypercapnic acidosis down to pH 7.0 (Gesser and Poupa, 1979). After an initial reduction, contractility recovered to ~90% of control level and was maintained for 120 min, probably due to an elevation of intracellular Ca^{2+} (Gesser and Poupa, 1981). Another example of tolerance to acidosis is provided by the European eel (*Anguilla Anguilla* L.), which in nature experience severe respiratory and metabolic acidosis during excursions into air when migrating between lakes (McKenzie et al., 2002). Eels exposed to graded hypercapnic acidosis from pH 7.9 to below 7.2, displayed an unchanged cardiac output as a result of an increased stoke volume. Like for the flounder, the cellular mechanism underlying this phenomenon is believed to be an elevation of intracellular Ca^{2+}.

Clearly, the results from the present study indicate that the crucian carp heart, like that of the flounder and eel, is tolerant of acidosis when oxygen is available. The ability to tolerate acidosis can be based on at least three compensatory mechanisms (Farrell, 1984): (1) H^+ extrusion from the myocardium mainly by the Na^+-H^+ exchanger and the Na^+-HCO_3^- symport, (2) intracellular buffering by HCO_3^- and non-HCO_3^- buffers, and (3) intracellular mobilization of Ca^{2+} mainly by reverse-mode NCX. Little is known about the cellular strategy of crucian carp cardiomyocytes, but the basis for cardiac acidotic tolerance in ectotherms may be the plasticity of the cardiac AP that is shaped by acidosis (Stecyk et al., 2008).

As a consequence of the anoxic crucian carp's ability to convert lactate to ethanol the anaerobic glycolysis can go on over prolonged time without accumulation of lactate and H^+ ions. Previous measurement of the extracellular pH of anoxic crucian carp has showed an initial decrease followed by a stabilization close to pH 7.4 after 24 h in 15^0C (van den Thillart and van Waarde, 1991; Van Waarde, 1991). This probably means that the anoxic crucian carp heart normally never experience an extracellular pH below 7.4. Thus, the finding that the crucian carp heart can tolerate acidosis below pH 7.4 during normoxia suggests that there is a

redundancy in the acidity tolerance of the crucian carp heart.

4.2.2 Heart response to anoxia

The typical response of the teleost cardiac tissue to oxygen deprivation is a reduction of cardiac force production, although the relative magnitude of the effect varies among species (Driedzic and Gesser, 1994; Farrell et al., 1992; Gesser, 1977; Poupa, 1976; Turner and Driedzic, 1980). The negative effect of oxygen deprivation arise because anoxia inhibits excitation-contraction coupling (Nielsen and Gesser, 1984) and contractile proteins (Matthews et al., 1986), elevates intracellular inorganic phosphate, which decreases Ca^{2+} sensitivity of the myofilament (Gesser and Jørgensen, 1982), and modifies myocardial action potential shape (Stern et al., 1988).

In agreement with the findings for other teleost species, anoxia-exposed (constant pH 7.8) crucian carp hearts exhibited an approximate 25% decrease of F_{max} after 20 min of exposure (Fig. 6). Additionally, the anoxic crucian carp hearts exhibited reductions in f_H, PC, TPT and $T_{1/2R}$. Thus, in general, the AC group appeared to suppress cardiac performance to a lowered new "steady state". This finding suggests that the crucian carp heart, when working in isolation, reduces its energy use, possibly in order to match ATP consumption with glycolytic ATP production. This may also function to protect the heart from intracellular acidosis, as it will allow more energy to be channeled to ion-pumps and calcium mobilization.

In vivo, the immediate cardiac response of crucian carp to anoxia is a strong reflex bradycardia and reduced cardiac contractility that is mediated by cholinergic receptors on sino-atrial pacemaker cells and atrial myocytes (Stecyk et al., 2004; Vornanen and Tuomennoro, 1999). The present findings indicate that there may also be an intrinsic component to the reduction of cardiac performance with anoxia exposure. However, with prolonged anoxia exposure, *in vivo* cardiac performance returns to the control normoxic level (Stecyk et al., 2004). This suggests that *in vivo*, the heart is stimulated by catecholamines, which enhance contractility by stimulating opening probability of calcium channels leading to an increase of activator calcium to out-compete the H^+ ions for troponin binding in the contractile apparatus (Gesser and Poupa, 1983). Indeed, pharmacological blockade of β-adrenergic receptors in anoxic crucian carp result in a decreased f_H. In the present experiments, adrenaline concentration was maintained at 1 nmol l^{-1}, and any additional sympathetic modulation could of course occur.

4.2.3 Heart response to combined anoxia and graded acidosis

Hearts exposed to graded acidosis during anoxia (the AA group) performed identically to the AC group during the first 60 min of exposure despite being exposed to pH 7.6 between 20 and 40 min and pH 7.4 between 40 and 60 min. This result was not surprising as the crucian carp in vivo survives prolonged anoxia with an extracellular pH of 7.4 (van den Thillart and van Waarde, 1991). The first aim of this thesis was to test the hypothesis that the combination of anoxia and extracellular pH below 7.4 is detrimental to crucian carp heart performance. The hypothesis was confirmed by the AA group exposed to pH 7.0 and 7.2, which exhibited a large fall in f_H and large increase in TPT and $T_{1/2R}$ compared to the AC group. This shows that although anoxia *per se* is well tolerated, leading to a new lower "steady state" level of activity, it is severely affected by a fall in pH below the normal anoxic level of 7.4. In nature, this reduced heart performance may not be sufficient to supply the needs of the brain and body for nutrition and waste transport in spite of adrenergic control. However, as discussed above, increased adrenergic cardiac stimulation during anoxia may counteract the depressive effect of low pH and anoxia on the anoxic crucian carp heart. More experiments are needed to test this hypothesis.

4.3 *Experiment 2*

The performance of the isolated crucian carp heart was compared to that of its anoxia sensitive relative, the koi carp. Heart performance in response to anoxia was compared at three different levels of pH, whereupon the hearts were allowed a period of recovery under the initial control conditions.

4.3.1 Response to 40 min anoxia at different levels of pH

Both crucian carp and koi carp exposed to 40 min of anoxia at any pH tested exhibited a significant reduction in PC, which arouse largely because of a slowing of f_H (Fig. 12). Interestingly, F_{max} of crucian carp heart decreased significantly from comparable NC hearts when exposed to anoxia at pH 7.4, but not when exposed to anoxia at pH 7.0, while the opposite response was seen in the koi carp. Also interestingly, in contrast to the results of *Experiment 1*, f_H did not differ between crucian carp heart exposed to anoxia at pH 7.4 and pH 7.0. These findings may represent differences in the experimental protocol or indicate a protective mechanism in crucian carp heart that is more effective at pH 7.0 than pH 7.4. This

could involve elevation of buffer capacity (Gesser, 1977) or that the sarcolemmal proton gradient becomes more energetically favorable in myocytes at pH 7.0 than 7.4 (Koop and Piper, 1992). It is possible that future measurements of intracellular pH in crucian carp myocytes exposed to graded acidosis may reveal ion channel activity that is more active at an extracellular pH of 7.0 than at a higher pH.

Another explanation for the similarity of f_H and F_{max} in anoxia exposed crucian carp hearts despite exposure to different pH, is that the 40 min exposure time was too short to trigger the change that was seen at the considerably longer and more gradual acidosis exposure utilized in *Experiment 1*. The anoxic exposure time of 40 min was chosen as a compromise between the anoxia tolerant crucian carp and anoxia intolerant koi carp hearts to ensure suitable comparison between the two species. It would not have been possible to lengthen the anoxia exposure as koi carp hearts would have likely been even more severely damaged. As the koi carp hearts were not even able to successfully recover F_{max} and PC after the 40 min of anoxia, a longer exposure time was not run in the present study. A future experiment could examine the crucian carp's hearts ability to successfully recovery after longer anoxic exposures below pH 7.4.

4.3.2 Response to 40 min of reoxygenation at pH 7.8

After 40 min of reoxygenation, f_H of both species recovered at all pH tested. Interestingly, only crucian carp also recovered F_{max} and PC at all pH used. These findings imply that neither pacemaker cells nor contractile function of crucian carp hearts were not irreversible damaged by anoxia and acidosis. The recovered f_H of the koi carp did not coinside with a complete recovery of F_{max}. Thus, unlike the crucian carp hearts, the contractile apparatus of koi carp hearts appeared irreversibly damaged by anoxia exposure, regardless of the extracellular pH. This also suggests that the koi carp heart heart may be more sensitive to anoxia than to acidosis. The catastrophic damage to koi carp could be a result of reperfusion injury (Zhang et al., 2008) or the stimulation of apoptotic pathways (Graham et al., 2004) in addition to the negative effects of anoxia on the intrinsic functioning of the heart.

In contrast to the findings for rate and force of contraction, contraction kinetics (TPT and $T_{1/2R}$) of crucian carp hearts did not fully recover after 40 min of reoxygenation, whereas they recovered in koi carp, though, during uncompleted contractions (Fig. 13). However, the pattern of change in these parameters suggests that both TPT and $T_{1/2R}$ would have recovered

to control levels if given a longer recovery period. In comparison, a similar pattern was not observed for F_{max} for koi carp hearts (Fig. 12D). This further supports the notion that the koi carp hearts were irreversibly damaged by the anoxia exposure.

4.4 Summary – Major findings and conclusions

The crucian carp is well known to tolerate anoxia, and the protective mechanisms allowing this includes ethanol production, which leads to a limited and stable reduction in extracellular pH to 7.4 (Van Waarde, 1991). In *Experiment 1*, crucian carp heart preparations exposed to anoxia and pH levels below 7.4, exhibited a decreased f_H and prolonged TPT and $T_{1/2R}$ suggesting that heart performance may not be able to meet the needs of the body if blood pH is not maintained above 7.4. In *Experiment 2* it was discovered that the performance of anoxic crucian carp hearts recovered during reoxygenation, implying that both pacemaker cells and contractile function was not irreversible damaged. In contrast, the heart of the anoxia-intolerant koi carp was not able to recover F_{max} and PC even after non-acidotic anoxia followed by reoxygenation. The koi carp heart showed no significant change in TPT and $T_{1/2R}$ except at pH 7.0, which is probably explained by an uncompleted contraction. This indicates that the contractile system in koi carp heart was more sensitive to anoxia than acidosis, except for the pacemaker cells. Probably, the ATP demand of the isolated koi carp heart could not be met by glycolytic energy production during anoxia. The crucian carp, on the other hand, is adapted to anoxia and its heart is able to maintain cardiac performance during anoxia, although when isolated from nervous input, its heart responds to anoxia by suppressing its activity.

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